Mechanism of Ribose 2′-Group Discrimination by an RNA Polymerase†

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ABSTRACT: The mechanism by which T7 RNA polymerase (RNAP) discriminates between rNTP and dNTP substrates has been characterized. During transcript elongation T7 RNAP uses rNTPs 70−80-fold more efficiently than dNTPs. Discrimination of the hydrogen-bonding character of the ribose 2′-substituent contributes a largely K_m-mediated factor of ∼20 to this preference for rNTPs. Discrimination of 2′-substituent H-bonding character appears to be made through a hydrogen bond to the hydroxyl group of tyrosine 639. This hydrogen bond makes little net contribution to either rNTP ground or transition state binding energy apparently because it is balanced by the energy of desolvation of the tyrosine hydroxyl. This mechanism may reflect a strategy to facilitate translocation by minimizing contributions from polymerase−NMP moiety interactions to NTP binding energy so as to minimize the affinity of the NTP binding site for the 3′-NMP of the product nucleic acid.

Bacteriophage T7 RNAP[1] is the most well characterized representative of a class of monomeric ∼100 kDa DNA-directed RNAPs (McAllister, 1993). The structural simplicity of this polymerase relative to cellular multisubunit RNAPs has facilitated studies of its structure and mechanism. Structural similarities between T7 RNAP and polymerases of distinct template and substrate specificities and mechanistic similarities with the multisubunit RNAPs imply that studies of this experimentally accessible molecule are relevant to our general understanding of polymerase structure and transcription mechanism (Sousa, 1996).

Mutation of T7 RNAP tyrosine 639 to phenylalanine leads to a gross deficit in rNTP/dNTP discrimination (Sousa & Padilla, 1995). Since this mutation does not markedly change the kinetic parameters for utilization of rNTPs (Bonner *et al*., 1992, 1994; Osumi-Davis *et al*., 1992, 1994; Sousa & Padilla, 1995), its effect is largely limited to enhancement of the polymerase's ability to utilize dNTPs. In order to understand the basis of this effect and to characterize the mechanism of rNTP/dNTP discrimination by this polymerase, we have characterized the utilization of rNTPs, dNTPs, and nucleotide analogs by mutants with different amino acids at positions 639, 635, and 641. Our results suggest a mechanism of ribose 2′-group discrimination which avoids making a net contribution to NTP binding energy.

MATERIALS AND METHODS

Mutant Enzyme Preparation. Mutants were constructed by the method of Kunkel *et al*. (1991) using constructs, expression, and purification procedures described previously (Bonner *et al*., 1992). 2′-Derivatized NTPs were prepared and characterized as described previously (Aurup *et al*., 1992).

Relative Activity and rNTP/dNTP Selectivity Assays. Activity was determined in assays with either supercoiled pT75 (Tabor & Richardson, 1985) which contains the T7 *ϕ10* promoter, pBS (Strategene Corp.) which contains a single class III T7 promoter, or poly(dC) as templates. Reactions were carried out in 40 mM Tris-HCl, pH 8.0, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM DTT, and 10^{-7} M pT75 or pBS or 0.1 mg/mL poly(dC), and a single α-^{32}P-labeled NTP was added for labeling (typically 1 μL of 10 mCi/mL; 3000 Ci/mmol label was added for each 100 μL of reaction mix). Reaction volumes were typically 50 μL, and reactions were carried out in Falcon microtiter dishes. Prewarmed (37 °C) reactions containing all other reaction components were initiated by addition of 5 μL of polymerase to a final concentration of 10^{-8}−10^{-9} M (higher concentrations of the most poorly active mutants were used; relative activity values in the tables are corrected for the differences in polymerase concentration). Duplicate 4 μL aliquots were taken at 2.5, 5, 10, and 20 min and spotted onto DE81 filter paper which had been presoaked in 0.1 M EDTA and dried. To correct for pipetting error, radioactivity in the spotted aliquots was first quantitated with a phosphorimager (Molecular Dynamics Corp.), following which the filters were extensively washed with 0.5 M NaH_2PO_4, followed by washes with distilled water and 100% ethanol to remove unincorporated NTP. After washing the filters were again quantitated by phosphorimager. Incorporation rates were determined by fitting to percentage incorporation using time ranges over which incorporation was linear. rNTP/dNTP selectivity was determined by measuring the rate of incorporation of a labeled rNTP or the corresponding dNTP in reactions containing four unlabeled rNTPs. Incorporation of ddNTPs or 3′-dNTPs was evaluated by measuring the inhibition of incorporation of labeled NTP in reactions containing four rNTPs with varying concentrations (2.5−0.01 mM) of a single ddNTP or 3′-dNTP. Utilization of NTP analogs was evaluated by

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[1] Abbreviations: RNAP, RNA polymerase; DNAP, DNA polymerase; KF, Klenow fragment; RT, reverse transcriptase; NTP, nucleoside triphosphate; rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate; pol, polymerase.

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comparison of the relative rates of labeled NTP incorporation in reactions carried out as described but in which one of the four cognate rNTPs was replaced with an analog.

Determination of Kinetic Parameters. Rates as a function of [NTP] were determined as described above, except that the concentration of the NTP whose K_m was being evaluated was varied from 2.5 mM to 5 μM in serial 2-fold dilutions. Eadie−Hofstee plots were used to evaluate the rate data. pT75 (initially transcribed sequence: "GGGAGACCGGAAUU") was used to evaluate K_m for UTP and CTP and corresponding analogs; pBS (initially transcribed sequence: "GGGCGAATTGG") was used to evaluate K_m for ATP and corresponding analogs. Poly(dC) (with the addition of 0.5 mM GMP to suppress contributions from the K_m of the initiating nucleotide) was used to evaluate K_m for GTP. To evaluate the effect of water concentration on NTP K_m, kinetic parameters were determined in the presence of 0, 28%, or 55% (w/v) sucrose or glycerol. In these experiments the radiolabeled NTP was the same as the NTP whose K_m was being determined but was present in constant amounts in reactions with different NTP concentrations so that its specific activity was greatest in the reactions with the lowest NTP concentrations. This increased the absolute signal in reactions with the lowest incorporation rates. In 55% glycerol or sucrose V_{max} for the reactions was reduced ~10-fold relative to <30% glycerol/sucrose, and in 77% glycerol or sucrose V_{max} was reduced ≥~100-fold and K_m determinations became unreliable. For this reason K_m values were measured over only a 2-fold range of [H_2O]. K_m values determined at different [H_2O] concentrations were then fit to the equation $K_m = A[H_2O]^n$ (Dzingeleski & Wolfenden, 1993).

Determination of Kinetic Parameters for Dinucleotide Synthesis. Rates of dinucleotide synthesis were measured with T7 "hairpin" promoter templates of the sequence:

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 CACGCGAAATTAATACGACTCA
A  ||||||||||||||||||||      +1
ACAGCGCTTTAATTATGCTGAGTGATATCCTAG
   (HP-GG) (for "GG" synthesis)

 CACGCGAAATTAATACGACTCA
A  ||||||||||||||||||||      +1
ACAGCGCTTTAATTATGCTGAGTGATATCTGGA
   (HP-GA) (for "GA" synthesis)
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Synthetic DNAs were synthesized on an Applied Biosystems DNA synthesizer and RPC purified followed by annealing (heating to 80 °C in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl and slow cooling to room temperature) at a final concentration of 50 μM. Reactions with hairpin promoter templates were carried out as described for plasmid templates with promoters at a concentration of 10^{-9} M (for measurement of "rGrG" with wt and Y639F polymerase as well as for measurement of "rGdG" synthesis by Y639F) and at 10^{-8} M (for measurement of "rGrA" synthesis by Y639F and wt polymerases and "rGdG" synthesis by wt polymerase). Polymerases were at a concentration of 10^{-7} M to ensure saturation of the available promoters. All reactions contained 0.5 mM GMP and varying concentrations of GTP, dGTP, or ATP and the corresponding α-^{32}P-labeled NTP. Reactions were run at 37 °C, and aliquots were taken at 2.5, 5.0, and 10 min and electrophoresed on 20% acrylamide−4% bisacrylamide (a high bisacrylamide to acrylamide ratio was used to achieve separation of mono- and dinucleotides)−7 M urea gels in 1× TBE. Sample buffer contained 50% formamide and xylene cyanol but no bromophenol blue which otherwise caused a discontinuity in the electrophoretic pattern near the region where dinucleotides run. A phosphorimager was used to quantitate percent incorporation by measuring radioactivity in the dinucleotide and unincorporated mononucleotide bands. Eadie−Hofstee plots of rate data were used to evaluate NTP K_m and k_{cat}.

Band-Shift Assays. Band-shift assays were carried out as described in Diaz *et al*. (1996) with [γ-^{32}P]ATP end-labeled promoter that was identical in sequence to HP-GG but was fully double stranded.

RESULTS

Less Conservative Mutations of Y639 Show Reduced Activity but Not Reduced Discrimination Relative to Y639F. The Y639F mutant is, on average, ~20-fold less selective for rNTPs relative to dNTPs than the wt enzyme but retains an ~4-fold preference for rNTPs (Table 1). The possibility that the role of this side chain in rNTP/dNTP discrimination might involve formation of a hydrogen bond between the Y639 OH and the ribose 2′-OH was initially considered unlikely since elimination of such a bond might be expected to either increase rNTP K_m or reduce V_{max}, or both, depending on the contribution such a bond makes to either rNTP ground state or transition state binding, and yet the Y639F mutation markedly affected neither of these parameters. It was therefore speculated (Sousa & Padilla, 1995) that the reduced discrimination of Y639F might be due to reduced side-chain volume or local disruption of active site structure (perhaps limited simply to a shift in the position of the Y639 aromatic ring). Less conservative substitutions might then be expected to show reductions in the residual ~4-fold preference for rNTPs over dNTPs displayed by Y639F since such mutations would further reduce side-chain volume or cause larger changes in active site structure. To test this the rNTP/dNTP selectivity of all 19 possible substitutions at position 639 was examined (Table 1). None of these less conservative substitutions show less rNTP/dNTP selectivity than Y639F, and some of these less conservative substitutions actually enhance rNTP/dNTP selectivity relative to Y639F.

Less conservative substitutions at this position reduce polymerase activity markedly. Activity correlates positively with increasing hydrophobicity of the side chain at position 639, but the correlation is imperfect since some bulky, polar substitutions display higher activity than smaller, less polar side chains. Relative activity is best predicted by a function which incorporates both the bulk and hydrophobicity of the mutant side chain (Table 1). Examination of predicted side-chain conformations reveals that a third parameter is the expected positional coincidence of the mutant side chain with the aromatic ring in the wt enzyme (Figure 1). Since Y639F displays wt activity, these observations imply that the Y639 aromatic ring—but not the hydroxyl group—is important for activity. To determine if the mutations affected promoter binding, we carried out band-shift assays with a labeled T7 hairpin promoter. All of the Y639 mutants tested displayed promoter binding activity, and for most this activity was indistinguishable from that of wt enzyme though some mutants (Y639S, Y639I, or Y639K) apparently exhibited moderately lower affinity (Figure 2).

Table 1: Relative Activity and rNTP/dNTP Selectivity of Y639 Mutants

	activity on			selectivity[b]				
side chain at 639	plasmid	poly(dC)[c]	H + B index[a]	rATP/dATP	rCTP/dCTP	rGTP/dGTP	rUTP/dTTP	av
Tyr (Y)	100	100	174	121 ± 33	89 ± 13	60 ± 11	34 ± 8.5	76
Phe (F)	100 ± 16	104 ± 7.0	196	5.0 ± 1.1	7.5 ± 2.0	2.0 ± 0.41	1.8 ± 3.9	4.1
Met (M)	52 ± 7.5	63 ± 8.5	183	8.8 ± 1.5	8.5 ± 2.0	2.3 ± 0.41	2.5 ± 0.84	5.5
Leu (L)	43 ± 6.2	50 ± 7.5	181	8.2 ± 1.1	21 ± 4.7	12 ± 3.2	4.0 ± 0.97	11
Cys (C)	15 ± 5.6	15 ± 3.4	132	14 ± 2.3	14 ± 5.3	9.2 ± 2.3	6.6 ± 2.4	11
Val (V)	8.6 ± 4.7	8.2 ± 0.66	155	21 ± 3.9	21 ± 3.9	26 ± 5.0	11 ± 1.7	19
Thr (T)	1.3 ± 0.43	8.5 ± 0.85	110	7.1 ± 2.4	7.1 ± 2.4	4.9 ± 1.0	5.0 ± 0.98	6.5
Gln (Q)	1.0 ± 0.17	20 ± 5.8	122	5.3 ± 1.5	6.8 ± 1.8	3.5 ± 1.6	2.4 ± 1.4	4.5
His (H)	0.37 ± 0.04	11 ± 0.70	153	nd[d]	nd	nd	nd	nd
Ala (A)	<0.2	20 ± 3.3	92	nd	nd	nd	nd	nd
Asn (N)	<0.2	13 ± 0.15	100	nd	nd	nd	nd	nd
Gly (G)	<0.2	6.1 ± 1.4	62	nd	nd	nd	nd	nd
Ile (I)	<0.2	6.0 ± 0.95	184	nd	nd	nd	nd	nd
Ser (S)	<0.2	3.9 ± 0.20	82	nd	nd	nd	nd	nd
Glu (E)	<0.2	1.9 ± 0.18	104	nd	nd	nd	nd	nd
Pro (P)	<0.2	1.6 ± 0.71	101	nd	nd	nd	nd	nd
Arg (R)	<0.2	1.6 ± 0.23	146	nd	nd	nd	nd	nd
Lys (K)	<0.2	0.66 ± 0.065	108	nd	nd	nd	nd	nd
Asp (D)	<0.2	nd	93	nd	nd	nd	nd	nd
Trp (W)	nd	nd	225	nd	nd	nd	nd	nd

[a] Hydrophobicity + bulk index = 1.3(residue volume) − 33(relative free energy of residue in interior vs surface) (Miller *et al*., 1987). Activity was a function of both the hydrophobicity and bulk of the side chain at position 639. Divergence between activity and the hydrophobicity + bulk index (i.e., Y639I) appears largely attributable to divergence in positional coincidence between the mutant and wt side chains at this position (Figure 1) as assessed from preferred rotamer configurations modeled with the program O (Jones *et al*., 1993). [b] Ratio of the rate of incorporation of labeled rNTP vs corresponding dNTP in reactions with excess unlabeled rNTPs. [c] Poly(dC) is a "facilitative" template relative to the plasmid, allowing the activity of less active mutants to be measured (Bonner *et al*., 1994). All mutant enzymes, except Y639W, showed wt stability, confirming reports (Osumi-Davis *et al*., 1994) that a tryptophan substitution at this position is destabilizing. An activity of 100 on a plasmid template corresponds to incorporation of 2.6% label/min (52 μM NTP/min) in reactions containing 10^{-7} M template and 10^{-8} M polymerase and 0.5 mM each NTP. An activity of 100 on poly(dC) corresponds to incorporation of 22 μM NTP/min in reactions with 0.1 mg/mL poly(dC) and 10^{-8} M polymerase. [d] Not determined.

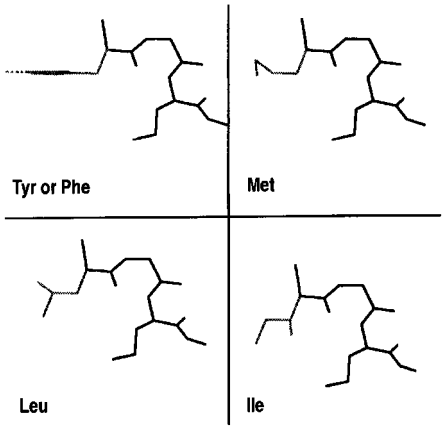

FIGURE 1: Preferred rotamer modeling of mutant side chains. Activity of T7 RNAP enzymes with different amino acids at position 639 correlates positively with hydrophobicity (Y639F,M,L,C,V were most active), but side-chain volume is an important secondary parameter (example: Y639Q was more active than Y639A, Table 1). As illustrated here another parameter is the expected positional coincidence of the mutant side chain with the aromatic ring of the wt enzyme. For example, Met, Leu, and Ile have similar volume and hydrophobicity, but the preferred rotamer of the methionine side chain is expected to coincide most closely with the wt tyrosine side chain (from the homologous KF structure; Beese *et al*., 1993). The leucine side chain is predicted to coincide less well, and the single rotamer of isoleucine points away from the plane of the aromatic ring. The activity of these three mutants is Y639M > Y639L ≫ Y639I.

Mutations at Y639 Eliminate Discrimination of the H-Bonding Character of the 2′-Ribose Substituent. The possibility that Y639 mutations create a "sloppy" active site which is indiscriminate with regard to NTP structure was tested by comparing utilization of base-modified NTPs (azido-ATP), α-thio-ATP, and 3′-deoxy-NTPs. These experiments did not reveal differences between the mutant and wt enzymes in discrimination of such modifications (Table 2), suggesting that the Y639F mutation specifically affects discrimination between a ribose and a 2′-deoxyribose. A

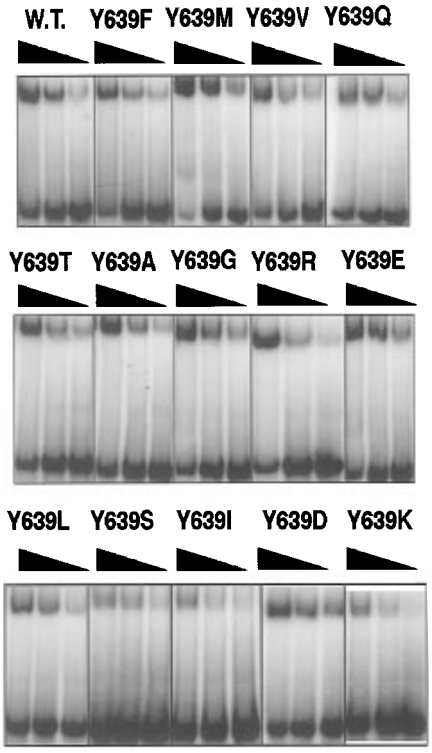

FIGURE 2: Band-shift assays carried out with the indicated polymerases at (from right to left in each panel) 6.25, 12.5, and 25 × 10^{-8} M, labeled T7 promoter (0.5 nM), and 25 μg/mL salmon sperm DNA.

Table 2: Relative Rates of Incorporation in Reactions with Base-, Ribose-, or Phosphate-Modified NTPs

polymerase	ATP/azido-ATP[a]	ATP/3′-dATP[b]	CTP/3′-dCTP[b]	ATP/ATPαS[a]
wt	4.0−5.5	3.9−4.4	2.8−3.6	1.0−1.3
Y639F	4.0−4.4	4.3−5.1	3.1−3.8	0.85−1.0

[a] Relative rates of incorporation in standard assays vs reactions in which ATP was replaced with azido-ATP or ATPαS (pT75 as template and reaction conditions as stated in Table 1). [b] Selectivity for the NTP vs the 3′-dNTP as determined by the concentration of 3′-dNTP required to reduce by 50% the yield of a defined sequence runoff transcript (*Hin*dIII linearized pT75 as template; reaction conditions as in Table 1) from $0.5 = (1 - [(3\text{-}'\text{dNTP}]/[\text{NTP}]S)^n$, where S is the selectivity and n is the number of times the NTP is used in synthesis of the transcript.

Table 3: Structural Characteristics of NTPs with Different Ribose 2′-Substituents

NTP	H-bonding character of 2′-group	volume (Å^3) of 2′-group	% ribose conformation[a] [% 3′-endo (N)]	electro-negativity ($^{\epsilon}R$)[b]
2′-F-NTP	weak acceptor	10.3	80	3.8−4.0
2′-OH-NTP	donor/acceptor	12.6	60	3.1−3.5
2′-H-NTP	none	5.5	40	2.1−2.2
2′-NH_2-NTP	donor/acceptor	17.5	20	2.6−3.1

[a] Percent contribution of 3′-endo (N) conformer to the equilibrium conformational mixture of NTPs with different ribose 2′-substituents as assessed by NMR. [b] Ranges in electronegativity values represent determinations by different investigators/methods (Gushlbauer *et al*., 1980; Saenger *et al*., 1984).

ribose and a deoxyribose differ in the character of their 2′-substituents as well as in the conformations, commonly expressed as the percent contribution of the 3′-endo (N) conformer to the rapidly interchanging equilibrium conformational mixture of the pentose (Saenger, 1984; Guschlbauer *et al*., 1980). To determine if discrimination of one or more of these structural features is reduced in the Y639 mutants, we examined utilization of NTPs with different substituents at the ribose 2′-position (Table 3). Previous studies of wt T7 RNAP utilization of pyrimidine nucleotides carrying these different ribose 2′-substituents revealed that the NTP preference of the enzyme followed the rank order 2′-OH > 2′-NH_2 > 2′-F (Aurup *et al*., 1992). Our observations for the wt enzyme accord with this (rank order 2′-OH > 2′-NH_2 > 2′-F > 2′-H); however, the Y639 mutants display a different rank order of NTP preference (2′-OH > 2′-F > 2′-H > 2′-NH_2; Table 4). Significantly, while some Y639 mutants display greater rNTP/dNTP discrimination than Y639F (Table 1), all the Y639 mutants tested display an identical rank order of NTP preference which is distinct from the wt enzyme.

To determine whether the discriminatory effects observed were mediated through K_m, V_{max}, or some combination of these parameters, we measured steady-state kinetic parameters for utilization of these NTPs by the wt and Y639F enzymes. Initially, we sought to develop a primer-extension assay analogous to that used in the characterization of DNAP enzymes since such an assay would eliminate the complications involved in evaluating kinetic parameters for a reaction with multiple phases (initiation/abortive cycling/elongation). While T7 RNAP can carry out primer-extension reactions (Cazenave & Uhlenbeck, 1994), we found that both simple primer-extension reactions and "transcription-bubble" RNA primer extensions (Daube & von Hippel, 1992) were inefficient and were plagued by nonspecific initiation and template extension ("clean" extension in these assays requires prelabeling of the primer only). An assay utilizing de novo initiation was therefore used, but it was critical, for simplicity in data interpretation, that this assay generate values for kinetic parameters that were dominated by the elongation phase of transcription during which RNAPs exhibit linear kinetics (Rhodes & Chamberlin, 1974; Kingston *et al*., 1981). Published studies of T7 RNAP suggest that this can be most readily achieved with supercoiled templates since (1) such templates do not limit transcript length, (2) the elongation complex is more stable on supercoiled templates, and (3) the initial transcribing complex is more stable and abortive cycling is minimized on supercoiled templates (Diaz *et al*., 1996). To test whether de novo initiated transcription assays would in fact give kinetic parameters dominated by the elongation phase of transcription, we carried out gel analysis of transcription reactions with NTP concentrations varied over the ranges to be used in determination of kinetic parameters (Figure 3). On supercoiled templates incorporation of NTP into short abortive transcripts was found to be a small percentage of that incorporated into long transcripts, even at limiting NTP concentrations and was found to be <0.2% (pBS, 10 μM CTP), <0.06% (pT75, 10 μM CTP), <0.02% (pT75, 5 μM UTP), <0.03% (pT75, 5 μM GTP), <0.2% [poly(dC), 5 μM GTP], and <0.1% (pBS, 5 μM ATP) (values were obtained by dividing radioactive label incorporation into abortive transcripts from the gels shown in Figure 3 by the incorporation into long transcripts at the given concentrations of limiting NTP). These values are upper limits because of the difficulty of quantitating the low levels of abortive transcripts generated on these templates, but they suggest (1) that total incorporation in these reactions will overwhelmingly reflect incorporation into long (non-abortive) transcripts and (2) that, given measured rates for T7 RNAP elongation (∼200 s^{-1}, Golomb & Chamberlin, 1975) and initiation (∼0.8 s^{-1}, Martin & Coleman, 1987; 1−5 s^{-1}, Jia *et al*., 1996), initiation will not significantly limit incorporation rates (with the possible exception of transcription on pBS with limiting CTP). These observations implied that incorporation rates with these templates as measured by DE81 filter retention could be used to obtain kinetic parameters dominated by the elongation phase of the transcription reaction.

The kinetic parameters obtained in these assays are summarized in Table 5. The rank order NTP preference of the wt enzyme suggests that the H-bonding character of the 2′-substituent is an important determinant of substrate specificity. The kinetic parameters reveal that discrimination of the 2′-substituent H-bonding character has a large K_m component, in accord with previous results (Aurup *et al*., 1992). Thus the K_m values for 2′-NH_2-NTPs are on average less than 2-fold larger than the K_s values for the corresponding NTP, but the K_m values for the 2′-F- or H-NTPs are on average ∼20-fold larger. The Y639F mutation nearly eliminates the K_m difference between NTPs whose 2′-substituents differ in hydrogen-bonding properties so that K_m (or K_s) values for NTPs with different 2′-groups are similar.

Structural Modeling Supports a Hydrogen Bond between the Y639 Hydroxyl and the Ribose 2′-OH. Structures of the Klenow fragment of DNAP I complexed with dNTP (Beese *et al*., 1993) and of RT complexed with primer-template (Jacobo-Molina *et al*., 1993) allow modeling of NTP in the

Table 4: Percent Activity of wt and Y639 Mutants with NTPs with Different 2′-Substituents[a]

NTP	wt	Y639F	Y639M	Y639V	Y639Q
UTP	100	95 ± 6.7	50 ± 1.2	nd	nd
2′-NH_2-UTP	5.9 ± 0.27	12 ± 0.41	3.6 ± 0.19	nd	nd
2′-F-UTP	3.1 ± 0.14	73 ± 2.6	23 ± 0.72	nd	nd
2′-dUTP	2.4 ± 0.11	46 ± 2.4	11 ± 0.46	nd	nd
CTP	100	103 ± 2.3	54 ± 3.9	8.8 ± 0.54	1.0 ± 0.05
2′-NH_2-CTP	34 ± 0.86	60 ± 2.5	21 ± 0.38	0.35 ± 0.023	0.097 ± 0.006
2′-F-CTP	3.4 ± 0.22	63 ± 3.1	47 ± 0.70	1.8 ± 0.092	0.46 ± 0.014
2′-dCTP	1.6 ± 0.16	57 ± 1.7	32 ± 1.1	0.44 ± 0.056	0.13 ± 0.008
ATP	100	96 ± 3.0	51 ± 1.3	8.3 ± 0.90	0.96 ± 0.035
2′-NH_2-ATP	18 ± 0.39	21 ± 0.75	0.92 ± 0.035	0.22 ± 0.035	0.066 ± 0.007
2′-F-ATP	6.6 ± 0.12	50 ± 1.4	9.7 ± 0.20	0.38 ± 0.023	0.30 ± 0.015
2′-dATP	2.7 ± 0.28	40 ± 1.3	3.2 ± 0.11	0.19 ± 0.023	0.10 ± 0.007

[a] Relative activity was determined with one of the rNTPs replaced with a dNTP or a 2′-modified NTP. The labeling of NTP was UTP (in reactions with 2′-modified CTPs or ATPs) or CTP (in reactions with 2′-modified UTPs). Data are from four independent experiments. Template and polymerase concentrations and percent activity are as given in Table 1.

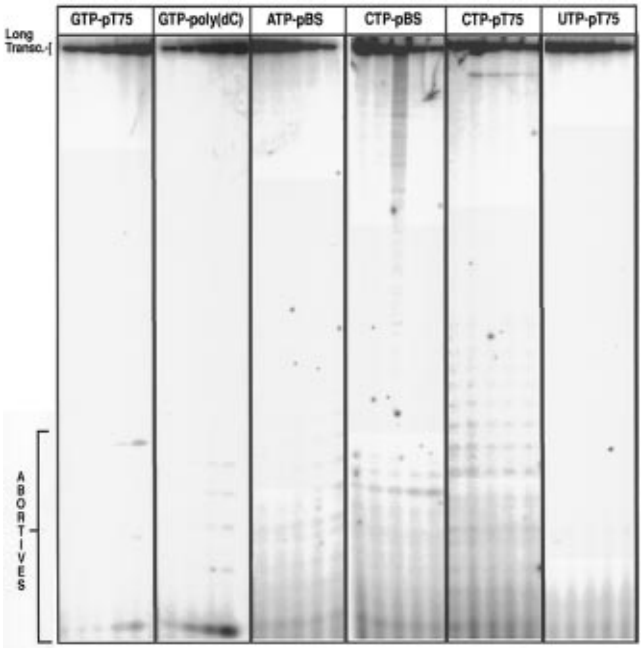

FIGURE 3: Patterns of long and abortive transcript synthesis as a function of [NTP] on the different templates used in this study. Plasmid templates (as indicated) and polymerases were at 0.1 μM and 2.5 nM; poly(dC) was at 0.1 mg/mL. Three NTPs were present at 0.5 mM in the reaction, and a single NTP (as indicated) was limiting and ranged from a concentration of 5 to 80 μM (ATP, GTP, UTP) or 10 to 160 μM (CTP) in serial 2-fold dilutions. In each panel NTP concentrations decrease from left to right. The radiolabeled NTP ([α-^{32}P]GTP) was present at constant (0.03 μM) concentration in each reaction, so that the specific activity of the label increases from left to right in the reactions with limiting GTP. Reactions were terminated at 10 min and electrophoresed on 20% acrylamide−4% bisacrylamide−7 M urea gels followed by quantitation with a phosphorimager to determine the relative incorporation into abortive vs long transcripts.

homologous, uncomplexed T7 RNAP structure (Sousa *et al*., 1993). Superposition of the T7 RNAP active site on the nonhomologous DNA polymerase β (Pelletier *et al*., 1994) following the superposition scheme proposed by Steitz *et al*. (1994) also allows NTP modeling in T7 RNAP. All these models (Figure 4) agree in placing the ribose close to Y639 so that a hydrogen bond with the 2′-OH and the Y639 OH is a possibility. Further, this modeling places no other hydrogen-bonding group within 5 Å of the ribose 2′-OH.

Utilization of Dideoxy-NTPs by the wt and Y639F Enzymes. Explaining the inefficient utilization of dNTPs by the wt enzyme in terms of a simple requirement for a hydrogen bond between Y639 and the ribose 2′-OH appears incompatible with the observation of efficient NTP utilization by Y639F. A more complex mechanism to explain our observations was therefore sought. For example, it is possible that the mechanism of discrimination involves competition between Y639 2′-OH and Y639 3′-OH hydrogen bonds such that, when the wt enzyme binds a 2′-dNTP, a hydrogen bond to the 3′-OH is established, causing the substrate to be bound with poor geometry for catalysis. The Y639F mutation might thereby exert its effect by eliminating the possibility of forming this inappropriate hydrogen bond. If this were the case, the wt enzyme might be expected to utilize dideoxy-NTPs with high efficiency. This was tested, and while both the wt and Y639 mutants can use 3′-deoxy-NTPs and the mutant enzyme can also use dideoxy-NTPs, the wt enzyme cannot efficiently utilize dideoxy-NTPs (Figure 5).

Table 5: Kinetic Parameters for NTP Utilization by wt and Y639 Mutants

	wt		Y639F	
NTP	K_m[a] (K_s)[b]	V_{max}	K_m (K_s)	V_{max}
UTP	32 ± 9 (128)	2.1 ± 0.14	28 ± 17 (112)	2.0 ± 0.31
2′-NH_2-UTP	120 ± 19	0.21 ± 0.006	89 ± 15	0.25 ± 0.02
2′-F-UTP	2800 ± 530	0.14 ± 0.02	190 ± 53	1.7 ± 0.21
2′-dUTP	1700 ± 330	0.05 ± 0.005	180 ± 67	1.0 ± 0.15
CTP	75 ± 39 (300)	2.0 ± 0.62	105 ± 26 (420)	2.2 ± 0.29
2′-NH_2-CTP	190 ± 58	0.60 ± 0.10	200 ± 78	0.95 ± 0.19
2′-F-CTP	3200 ± 460	0.27 ± 0.03	240 ± 48	1.3 ± 0.11
2′-dCTP	2000 ± 1400	0.11 ± 0.05	350 ± 36	1.1 ± 0.05
ATP	32 ± 18 (128)	2.1 ± 0.30	36 ± 20 (144)	2.2 ± 0.21
2′-NH_2-ATP	470 ± 190	0.61 ± 0.12	350 ± 68	0.54 ± 0.07
2′-F-ATP	3900 ± 3100	1.2 ± 0.74	320 ± 46	1.3 ± 0.11
2′-dATP	6400 ± 2000	0.93 ± 0.14	470 ± 97	1.1 ± 0.15

[a] K_m is in μM and is derived directly from Eadie−Hofstee plots and corresponds to [S] which gives $V = 0.5V_{max}$. [b] $K_s = K_m/f_s$, where f_s is the frequency of the complementary base in the template, assuming k_{cat} for utilization of each NTP is nearly identical (Rhodes & Chamberlin, 1974; Kingston *et al*., 1981). For dNTPs and analogs, the latter assumption cannot hold and k_{cat} for a dNTP or analog cannot be derived directly from the reductions in V_{max} (given in 0.1 μM NTP/s) since these involve both a reduction in incorporation of a dNMP or analog and subsequent extension of the 3′-dNMP or analog containing nucleic acid. For this reason, K_s values for the dNTPs and analogs are not derived; however, to the extent that a dNTP or analog is utilized more poorly than the rNTP, its incorporation becomes rate limiting and K_m is a close approximation of K_s. Reactions contained three rNTPs and the indicated dNTP, rNTP, or analog. Labeling of NTPs is as in Table 3. Data are from four independent experiments. Template and polymerase are at 0.1 μM and 2.5 nM, respectively.

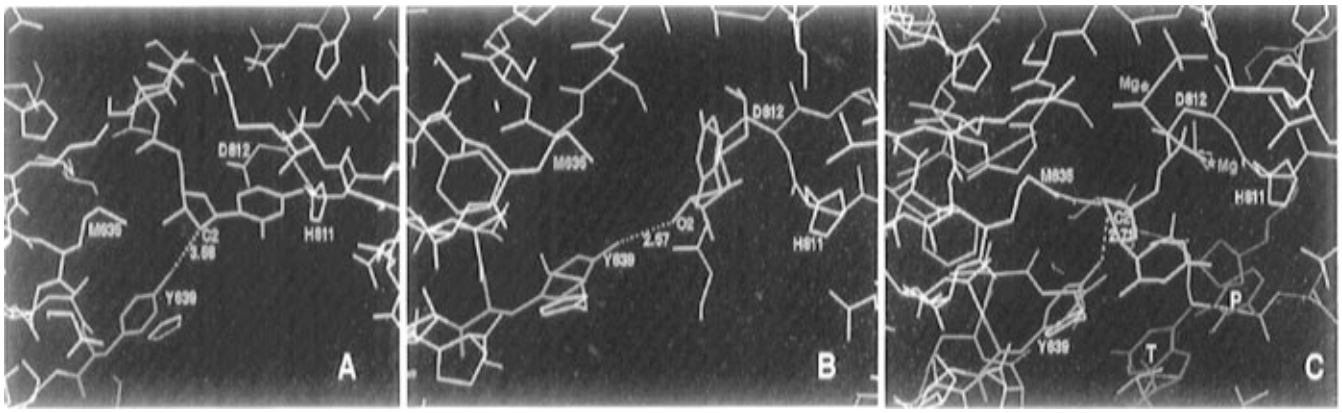

FIGURE 4: Modeling of NTP binding in T7 RNAP. (A) Location of an NTP in the T7 RNAP active site based on the Klenow fragment−dCTP structure (Beese *et al*., 1993). Side chains were modeled on the T7 RNAP α-carbon structure using KF side-chain conformations for conserved residues and preferred rotamers as modeled by the program O (Jones *et al*., 1993) for residues which differ between the two polymerases. Subsequently, the T7 RNAP and Klenow fragment−dCTP structures were manually superimposed using the program TOM to reveal where the dCTP might lie in the T7 RNAP structure. Superposition of the two structures was carried out so as to maximize the alignment between well-conserved structural elements in the two enzymes and to bring the α-carbons of the conserved, catalytic aspartic acids (KF D705/T7 RNAP D537; KF D882/T7 RNAP D812) into near superposition. Superposition of RT−RNAP (B) and DNAP β−RNAP structures (C) was done similarly to bring the α-carbons of the catalytic carboxylates and the β-strand structures which contain these carboxylates into alignment. (B) Location of an NTP in the T7 RNAP active site based on the RT primer-template structure (Jacobo-Molina *et al*., 1993). An rCMP was modeled into the RT primer-template structure by extending the primer-template by 1 base pair following A-form helix geometry. (C) Modeling based on the DNAP β primer-template ddNTP structure (Pelletier *et al*., 1994). In (C) the locations of the Mg^{2+} ions, the complementary template base (T), and the 3′-ddNMP of the primer (P) relative to the ddNTP are also shown.

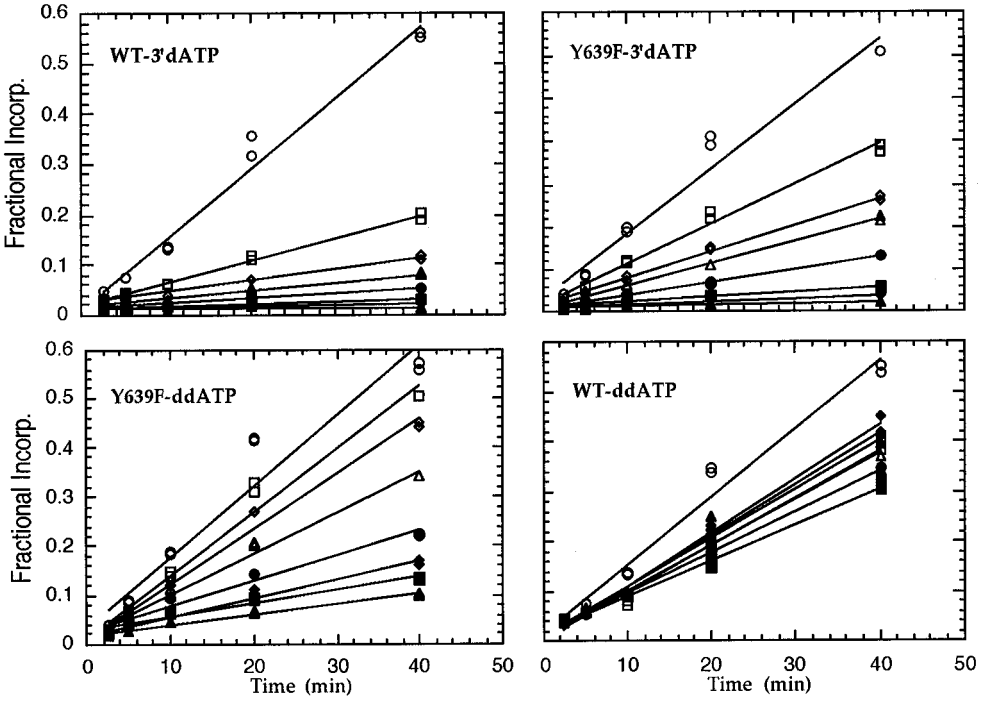

FIGURE 5: 3′-dATP inhibition of wt and Y639F activity. Incorporation by both wt and Y639F enzymes is effectively inhibited by the addition of increasing concentrations of 3′-dATP, and Y639F is also sensitive to inhibition by 2′,3′-ddATP but the wt enzyme is much less so. 3′-dATP concentrations (μM): open circles, 0; open squares, 0.5; open diamonds, 1; open triangles, 2; filled circles, 4; filled squares, 8; filled diamonds, 16; filled triangles, 32. 2′,3′-ddATP concentrations (μM): open circles, 0; open squares, 2; open diamonds, 4; open triangles, 8; filled circles, 16; filled squares, 32; filled diamonds, 64; filled triangles, 128.

Effects of Mutation of Other Active Site Side Chains on 2′-Group Discrimination. It is also conceivable that, in the absence of a bound rNTP, a hydrogen bond forms between Y639 and some other active site side chain. Examination of the active site reveals no side chains which could form hydrogen bonds within 5 Å of the Y639 OH so that, barring active site isomerization or substantial errors in side-chain modeling, this hypothesis appears untenable. The possibility of an interaction between M635 and Y639 was tested since M635 and Y639 are close and M635 approaches the ribose in the models (Figure 4). This position is methionine in the T7 RNAP class of RNAPs (McAllister, 1993) but is either Tyr or Phe in the homologous DNAPs, and in the DNAPs mutants at this site affect dNTP/ddNTP discrimination (Tabor & Richardson, 1995). However, while M635A, M635F, or M635Y mutants had effects on NTP K_m, they did not affect 2′-group discrimination in either a wt or Y639F background (Table 6). The report (Kostyuk *et al*., 1995) that mutation of S641 to alanine affected dNTP/rNTP discrimination was tested, but we found that the S641A mutation had no effect

Table 6: rNTP/dNTP Selectivity of M635 and S641 Mutants[a]

mutant	rATP/dATP	rCTP/dCTP	rGTP/dGTP	rUTP/dTTP	av
S641A	125 ± 28	77 ± 10	59 ± 7.4	30 ± 9.0	73
S641A/Y639F	12 ± 2.3	10 ± 4.2	4.1 ± 1.6	2.7 ± 0.57	7.3
M635Y	131 ± 17	81 ± 12	62 ± 5.1	28 ± 8.3	76
M635Y/Y639F	14 ± 3.0	11 ± 3.4	5.0 ± 1.2	3.1 ± 0.5	8.3
M635F	120 ± 11	88 ± 16	60 ± 9.4	32.0 ± 4.2	75
M635F/Y639F	6.2 ± 1.5	8.3 ± 1.2	3.5 ± 0.8	1.9 ± 0.3	5.0
M635A	>100	95 ± 23	83 ± 18	43 ± 11	>80
M635A/Y639F	17 ± 7.8	17 ± 5.2	19 ± 3.2	7.8 ± 2.5	15

[a] Polymerase and template concentrations are as in Table 1. ± values express ranges from two experiments.

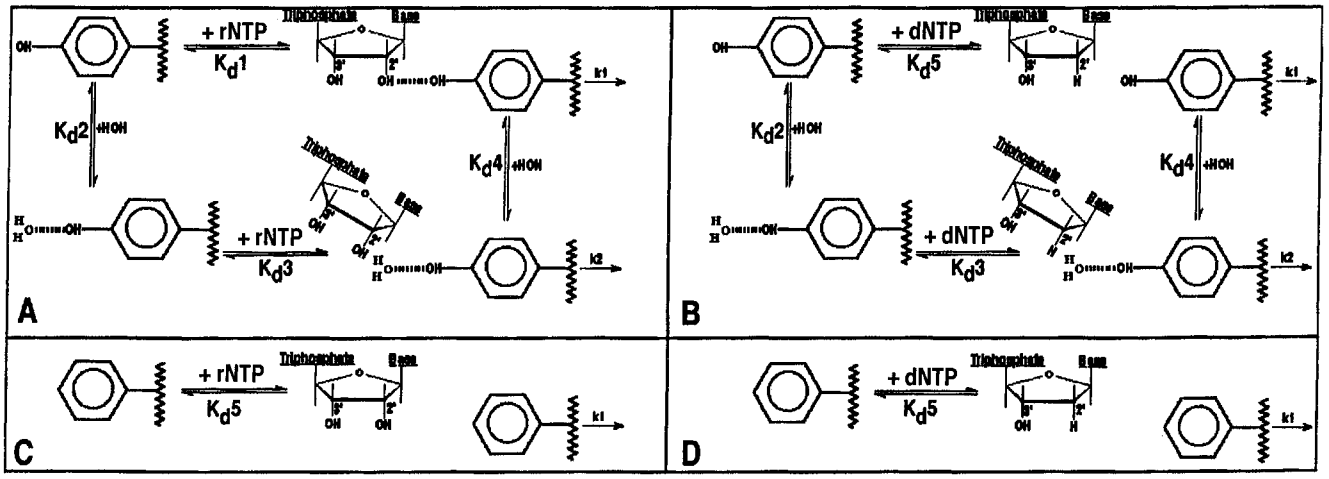

FIGURE 6: Kinetic schemes for rNTP (A) or dNTP (B) binding to the wt enzyme or to Y639F (C, D), respectively. For illustrative purposes the 2′-group−Y(or F)639 interaction is isolated (i.e., effects due to ribose conformation, substrate/polymerase desolvation at other positions, etc. are not considered). Catalytically optimal binding requires displacement of a Y639 H-bonded water in (A) or (B) and is associated with $k_1 > k_2$, but we do not set $k_2 = 0$ since a low catalytic rate when NTP is bound with poor geometry cannot be formally excluded. We assume that the NTP binding constant (K_d5) is similar in (B), (C), and (D) but is less (K_d1) in (A) since in the latter an additional hydrogen bond with the substrate is established. However, the competitive effect of the water causes the apparent K_m in (A) and (B) to be greater than the corresponding K_d. Effects on V_{max} also arise; for (B) the rate equation is $v = k_1[E_{tot}][NTP](1 + k_2[H_2O]/k_1K_d4)/([NTP](1 + [H_2O]/K_d4) + K_d5(1 + [H_2O]/K_d2))$ (Dixon & Webb, 1979).

on discrimination (Table 6). A number of other side chains near the modeled ribose (H811, D812; Figure 4), base, or phosphates have been previously found to have no effect on dNTP/rNTP discrimination (Sousa & Padilla, 1995). Therefore, involvement of another side chain in the 2′-group discrimination mediated by Y639 appears unlikely.

Effects of Changes in Water Concentration on the Relative K_m Values of Y639F and the wt Enzyme for an rNTP. Another possibility is that a water molecule binds to the tyrosine OH and that catalytically optimal binding of NTP requires displacement of this water molecule as illustrated in Figure 6. The mechanism presented in Figure 6 predicts that the affinity of Y639F for rNTP should decrease, *relative to the wt enzyme*, as water concentration is reduced, and this change in relative affinities should be reflected in K_m to the extent that K_m reflects K_d. If NTP binding obeys Michaelis−Menten kinetics, then log K_m should vary linearly with N log [H_2O], where N is the number of water molecules released upon substrate binding (Dzingelski & Wolfenden, 1993). We therefore measured NTP K_m for the wt enzyme and Y639F as a function of water concentration. As shown in Table 7 and Figure 7, K_m values in fact vary with water concentration as predicted by a mechanism in which discrimination is mediated by 2′-OH displacement of a water molecule bound to the Y639 OH, and the difference value—$N_{Y639F} - N_{wt}$—is close to 1 for all four NTPs. This indication of rapid equilibrium kinetics is consistent with observations that T7 RNAP is homologous to polymerases where NTP binding follows rapid equilibrium kinetics (Patel *et al*., 1991; Dahlberg & Benkovic, 1991) and the isolation of T7 RNAP mutants that affect rates but not K_m (Bonner *et al*., 1992, 1994; Osumi-Davis *et al*., 1992, 1994).

Kinetic Parameters for Dinucleotide Synthesis. Derivation of kinetic parameters from incorporation assays involving de novo initiated transcripts is complicated by the existence of initiation, abortive cycling, and elongation phases in the transcription reaction. While analysis (Figure 3) revealed that use of appropriate templates allows such reactions to be dominated by the elongation phase of transcription, we also chose to evaluate parameters for a kinetically much simpler reaction: formation of the first phosphodiester bond in initiation (dinucleotide synthesis) on partially single-stranded hairpin promoter templates. The promoter−polymerase complexes formed on such templates are stable (dissociation rates ~0.02/s^{-1}; Jia *et al*., 1996) as are the initially transcribing complexes (half-life >30 min; Diaz *et al*., 1996) so that, when either polymerase or promoter is saturating and the polymerization reaction is limited to dinucleotide synthesis (by NTP limitation), the rates of promoter−polymerase dissociation should not impact on kinetic parameters for dinucleotide synthesis. GMP has been shown to be used as efficiently as GTP as the initiating nucleotide (Martin & Coleman, 1989) but cannot be used as an elongating substrate. Further, the K_m for GMP as an initiating substrate is much lower than its K_i for inhibition of GTP utilization as an elongating substrate (Martin &

Table 7: Effects of Varying [H_2O] on NTP K_m

H_2O (M)	K_m (wt)	K_m (Y639F)	$N_{wt} - N_{Y639F}$[a]
	UTP (Sucrose)		
55	41 ± 5.7	27 ± 3.2	
41	43 ± 12	38 ± 5.5	
27	32 ± 4.3	52 ± 9.4	
			1.0 ± 0.23
	ATP (Sucrose)		
55	36 ± 6.8	24 ± 4.0	
41	35 ± 3.0	32 ± 2.6	
27	37 ± 6.5	54 ± 8.4	
			1.1 ± 0.20
	GTP (Sucrose)		
55	52.8 ± 5.0	55.0 ± 4.0	
41	39.2 ± 3.2	93.9 ± 8.4	
27	42.1 ± 5.4	111.7 ± 2.6	
			1.3 ± 0.28
	CTP (Sucrose)		
55	122 ± 11	110 ± 9.2	
41	77 ± 6.8	113 ± 11	
27	62 ± 6.1	106 ± 5.6	
			0.98 ± 0.13
	UTP (Glycerol)		
55	46 ± 10	27 ± 4.2	
41	56 ± 7.6	32 ± 2.2	
27	36 ± 7.6	42 ± 1.0	
			1.0 ± 0.12

[a] Variations in K_m as a function of [H_2O] were fit according to Dzingelski and Wolfenden (1993) where water is treated explicitly in binding: $E{\cdot}nH_2O + NTP \rightleftharpoons E{\cdot}mH_2O{\cdot}NTP + (n - m)H_2O$; so $K_d = ([E{\cdot}nH_2O][NTP])/([E{\cdot}mH_2O{\cdot}NTP][H_2O]^{n-m})$. When [NTP] = K_m, $[E{\cdot}nH_2O] = [E{\cdot}mH_2O{\cdot}NTP]$ (the case of a homopolymer template is treated for simplicity) so $K_m = K_d[H_2O]^N$, where $N = n - m$ (Figure 7). The *absolute* value of N cannot be predicted a priori since an unknown number of waters are displaced or become associated with the enzyme upon NTP binding, and this number may vary for different NTPs, but the mechanism predicts $N_{wt} = N_{Y639F} + 1$ for all NTPs. Data are from an average of five independent experiments. Templates are at 0.1 μM (plasmids) or 1 mg/mL [poly(dC)]; polymerase is at 2.5 nM.

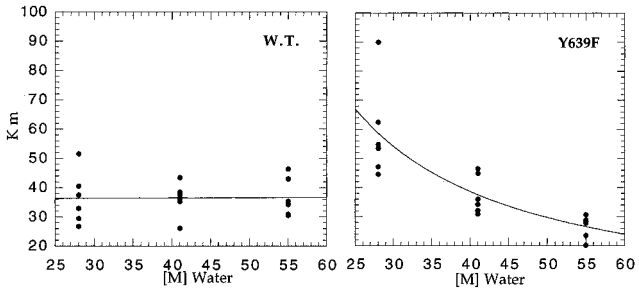

FIGURE 7: Representative determination of the effects of varying [H_2O] on apparent NTP K_m for the wt and Y639F enzymes. The data shown are for ATP (six independent experiments). The data are fit to $K_m = A[H_2O]^n$ and give $A = 35$, $n = 0.011$ and $A = 2458$, $n = -1.07$ for the wt and Y639F polymerases, respectively. The value n is interpreted as the net number of water molecules which become associated with the complex upon substrate binding (Dzingeleski & Wolfenden, 1993).

Coleman, 1989; Guajardo & Sousa, 1997). Therefore, when GMP is added to dinucleotide synthesis reactions at concentrations which nearly saturate the initiating substrate binding site of the polymerase, kinetic parameters for utilization of the elongating substrate can be measured.

While kinetically simpler than extended transcript synthesis, the NTPs which can be used in this assay are limited by the inefficiency of the initiation reaction relative to the elongation reaction (some ~200-fold slower; Martin & Coleman, 1987) and its sensitivity to NTP structure. Promoters that initiate "GA" are significantly less active than the canonical "GG" initiating promoter, and "GC" or "GU" initiating promoters are even less active (Milligan *et al.*, 1987). This constrained our use of this assay to measuring kinetic parameters for utilization of GTP, ATP, and dGTP as elongating substrates (Figure 8 and Table 8). Utilization of dGTP by the wt enzyme was associated with a very high K_m relative to GTP utilization, but Y639F displayed a similar K_m for both substrates. It was also found that the presence of 55% sucrose reduced K_m for rGTP utilization by the wt enzyme but not by Y639F. The utilization of ATP by either the wt or Y639F enzyme in this assay was associated with a much higher K_m and lower k_{cat} than the utilization of GTP, and there was also a much larger difference in k_{cat} for utilization of dGTP (by either Y639F or the wt enzyme) in these assays than in the plasmid-based assays. Utilization of dATP was too poor to measure accurately even with Y639F polymerase.

DISCUSSION

We find that T7 RNAP displays an rNTP/dNTP specificity factor of 70−80. The modest specificity of T7 RNAP contrasts with that of DNAPs (Tabor & Richardson, 1989) but is consistent with reports that wt T7 RNAP can be used to incorporate dNMPs into transcripts (Aurup *et al.*, 1992). Such modest specificity may reflect a higher in vivo concentration of rNTPs than dNTPs (Arezzo, 1987), requiring in turn a higher degree of substrate specificity for DNAPs than RNAPs in order to maintain similar homogeneity in their respective products, and it may also mean that infrequent incorporation of dNMPs into transcripts is less detrimental than incorporation of rNMPs into DNA.

Most (a factor of ~20) of this specificity is lost upon mutation of Y639 to phenylalanine. We draw the conclusion that removal of the OH group is directly responsible for the 20-fold loss in substrate specificity since less conservative mutations cause no further reductions in the residual specificity displayed by Y639F (Table 1). Such reductions might be expected if the effect of removal of the OH group were due to a reduction in side-chain volume or local disruption in active site structure. Lack of disruption of active site structure in the Y639F mutant is indicated by the observation that, though the aromatic ring is important for activity as shown by the effects of less conservative substitutions, the Y639F mutant displays wt kinetic parameters with rNTPs suggesting that the aromatic ring is similarly positioned in Y639F and the wt enzymes. With regard to substrate specificity it is significant that, while some less conservative substitutions display increased specificity relative to Y639F, all the mutants tested show the same rank order preference for differently 2′-modified NTPs, a rank order preference which is distinct from that of the wt enzyme (Tables 4 and 5) and which apparently reflects loss of a largely K_m-mediated discrimination of the ribose 2′-group H-bonding character.

Structural modeling of NTP binding by T7 RNAP based on KF−dNTP, RT−primer-template, and DNA pol β−primer-template−ddNTP complexes is consistent with the existence of a hydrogen bond between the ribose 2′-OH and the Y639-OH (Figure 4), but this modeling requires further comment. The KF−dNTP complex is a noncatalytic binary complex

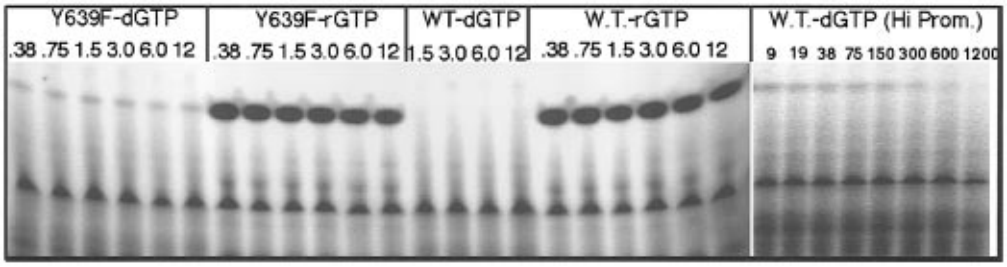

FIGURE 8: Dinucleotide synthesis assay. Reactions were carried out with the indicated polymerases and NTPs at the indicated concentrations. Promoter and polymerase concentrations were 10^{-9} and 10^{-7} M, respectively, except in the panel labeled "Hi Prom", where the promoter and polymerase concentrations were 10^{-7} and 1.7×10^{-6} M, respectively. GMP was present at 1 mM in all the reactions and [α-^{32}P]dGTP or [α-^{32}P]rGTP was present at a constant concentration (0.01 μM) in all the reactions so that the specific activity of the label decreases proportionately with increasing NTP concentration. The dinucleotide product is the upper band; the lower band is a contaminant of the radioactive NTP. Mononucleotide triphosphates have run off the gel. The gel shows the 10 min time point for these reactions.

Table 8: Kinetic Parameters for Dinucleotide Synthesis

	wt		Y639F	
NTP	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
GTP	17.5 ± 2.5	2.55 ± 0.15	10.35 ± 0.65	1.8 ± 0.1
dGTP	850 ± 76	0.018 ± 0.001	12 ± 0.70	0.054 ± 0.005
GTP (sucrose)[a]	10.3 ± 1.6	0.44 ± 0.09	11.5 ± 0.5	0.51 ± 0.11
ATP	286 ± 27	0.20 ± 0.06	250 ± 26	0.096 ± 0.003

[a] Sucrose concentration in these experiments was 55% (w/v) corresponding to ~28 M H_2O. Promoter and polymerase concentrations are as follows. For determination of kinetic parameters for GTP and GTP (sucrose), [promoter] = 10^{-9} M and [polymerase] = 10^{-7} M. For dGTP with Y639F, [promoter] = 10^{-9} M and [polymerase] = 10^{-7} M. For dGTP with wt, [promoter] = 10^{-7} M and [polymerase] = 1.7×10^{-6} M. For ATP, [promoter] = 2×10^{-7} M and [polymerase] = 2×10^{-6} M. Data are from three experiments.

and could be considered irrelevant to a catalytic complex (Beese & Steitz, 1993). However, the phosphate contacts observed in this complex have been confirmed by kinetic studies of appropriate site-directed mutants (Astatke *et al*., 1995) which also indicate a likely deoxyribose−F762 contact which supports the relevance of the binary complex in identifying the position, though not the base orientation, of the dNTP in the catalytic complex. The relevance of the binary complex is also supported by the observation that F762 has been identified as the critical residue in discrimination of the 3′-deoxyribose substituent (Tabor & Richardson, 1995). All available evidence therefore supports assignment of the C-terminal region of KF helix O (residues F762 to Y766) as a deoxyribose binding site with F762 closer to the 3′-OH and Y766 closer to the 2′-position. In T7 RNAP the corresponding residues are M635 and Y639. Further evidence for the relevance of the KF−dNTP complex in modeling the T7 RNAP−rNTP complex emerges from observations that mutation of M635 to alanine in T7 RNAP causes a large increase in K_m for GTP, but not PP_i, indicating that M635 establishes a contact with the NMP moiety of the NTP (Guajardo & Sousa, 1997). Similar results and conclusions were obtained when the corresponding residue in DNAP I (F762) was mutated (Astatke *et al*., 1995). Furthermore, modeling based on the RT and DNA pol β complexes is not subject to the criticism that these complexes are catalytically irrelevant, but since both of these polymerases are less homologous to T7 RNAP, the relevance of these complexes to T7 RNAP might be questioned on this basis, even though the current consensus is that all of these polymerases share a similar catalytic mechanism and positioning of the substrate relative to the catalytic carboxylates (Steitz *et al*., 1994). Ultimately, positioning of the NTP in T7 RNAP is limited by the accuracy of the modeling, and it is incorrect to suggest that modeling can conclusively establish the existence of a hydrogen bond between the Y639 OH and the ribose 2′-OH. However, barring gross inaccuracies in this modeling or substantial conformational changes, there does not appear to be any structurally reasonable mechanism that would allow discrimination of the H-bonding character of the 2′-ribose substituent except interaction with the Y639 OH since no other H-bonding group is placed within 5 Å of the 2′-OH and mutation of other residues predicted to be near the ribose shows no effect on 2′-group discrimination.

These observations are therefore consistent with the Y639 OH discriminating the hydrogen-bonding character of the ribose 2′-substituent through a direct hydrogen bond. While other mechanisms in which undefined changes in active site geometry are responsible for the observed effects cannot be excluded, it appears difficult to reconcile any other specific mechanism with the structural modeling, the observation that Y639 mutants specifically lose discrimination of 2′-group H-bonding character, and the wt kinetics of Y639F for rNTP utilization. To reconcile the latter observation with the hypothesis that Y639F eliminates an H-bond between enzyme and substrate, the mechanism elaborated in Figure 6 is proposed. In this mechanism a water molecule bound to the Y639 OH formally plays a role as a partial mixed inhibitor of NTP utilization: catalytically optimal NTP binding requires displacement of this water molecule. Through consideration of the net number of hydrogen bonds formed upon substrate binding, this mechanism explains the apparent paradox that elimination of the H-bonding potential at the ribose 2′-position causes an increase in K_m, while elimination of the putative H-bonding partner of this group (the Y639 OH) does not. Aspects of such a mechanism would have precedent. Fersht *et al*. (1985) showed that substrate binding to tyrosyl-tRNA synthetase mutated at position 51 was associated with a high K_m for the S51-substituted enzyme and a low K_m for the C51- or A51-substituted enzyme. The interpretation offered for these patterns was that substrate binding required displacement of a water molecule H-bonded to S51 or C51. Only in the latter case was the side chain in position to establish a compensating H-bond with the substrate, and therefore K_m was lower for the C51-substituted enzyme. For the A51 substitution such an H-bond to the substrate was not made, but neither was it necessary to displace an H-bonded water and K_m was also lower. Relative to the T7 RNAP discrimination

mechanism the synthetase C51−substrate interaction is analogous to the wt−rNTP interaction (low K_m), the A51−substrate interaction is analogous to the Y639F−rNTP/dNTP interaction (also low K_m), and the S51−substrate interaction is analogous to the wt−dNTP interaction (high K_m). It needs to be remarked that, even if we assume that the Y639 OH−2′-OH bond makes its full contribution to ground state binding, this mechanism does not predict that discrimination of 2′-group H-bond character will have *only* a K_m component. An effect on V_{max} emerges from the noncompetitive component (associated with catalytically nonoptimal NTP binding) of the kinetics illustrated in Figure 6; hence, the rank order to V_{max} values for wt T7 RNAP utilization of NTPs with different 2′-substituents cannot be expected to coincide with that of the Y639 mutants. Experimental support for this mechanism emerges from the changes in the rNTP K_m of the wt enzyme relative to Y639F as a function of water concentration which are found to be consistent with release of an additional water molecule upon rNTP binding to the wt enzyme vs Y639F (Tables 7 and 8, Figure 7), and from additional mutagenesis and modified NTP utilization experiments which argue against models in which another active site side chain (Table 6) or an alternate hydrogen bond to the ribose 3′-OH (Figure 5) is involved in 2′-group discrimination.

Since changes in water concentration are achieved by addition of cosolvents which change the viscosity and dielectric constant of the medium, it was important to determine if similar results would be obtained with cosolvents of different chemical structure which have differing effects on these parameters. The data in Table 7 with sucrose and glycerol as cosolvents are consistent with a mechanism which predicts water concentration to be the relevant variable. However, it is legitimate to ask why the effects on NTP K_m should be a function of water *concentration* rather than *activity*. Variations in kinetic parameters for a number of enzymes have been previously demonstrated to vary as a function of water concentration, rather than activity (Westhead & Malmstrom, 1955; Pocker & Janic, 1989; Dzingelski & Wolfenden, 1993), so the observations presented here are not without precedent. One explanation proposed for these observations is that the activity coefficients of the critical water molecules do not change during the reaction so that water concentration is left as the critical variable (Bell & Critchlow, 1971). Alternatively, water activity may be the more relevant parameter in our system, but use of water concentration may entail only second-order errors which are not detectable at our level of quantitative accuracy (Figure 7).

Though a tyrosine is conserved in the pol I class of DNAPs at the position corresponding to T7 RNAP Y639F, our current observations are at least superficially inconsistent with previous speculations that this side chain might play a similar role in substrate discrimination in both classes of enzymes. These speculations rested on the supposition that it is the aromatic ring of the tyrosine that is functionally critical in rNTP/dNTP discrimination and that shifts in the position of this ring (or other local changes in active site geometry) relax the substrate specificity of the enzyme (Sousa & Padilla, 1995). Such a mechanism could be operative in both RNAPs and DNAPs, but a mechanism in which the hydrogen-bonding properties of the OH group are utilized in discrimination may not be so readily extended to DNAPs. While it does not appear that the T7 RNAP mechanism immediately illuminates the mechanism used by DNAPs, MMLV RT uses a mechanism that is, in some sense, analogous to that of T7 RNAP. It has recently been shown that MMLV RT discriminates against rNTP substrates with an active site side chain (F155) that sterically clashes with the 2′-OH of an rNTP. Substitution of F155 with a smaller side chain relieves this steric clash and allows rNTP utilization (Gao *et al*., 1997). A simple steric clash like this could not be used to discriminate against dNTPs since the 2′-substituent is smaller in a dNTP than an rNTP; however, T7 RNAP apparently creates a steric clash for dNTP utilization by binding an obstructing water in the active site which is effectively displaced by a 2′-OH group but not by a 2′-H group, and in both MMLV RT and T7 RNAP relief of these steric clashes leads primarily to a reduction in K_m for the noncanonical substrate (Gao *et al*., 1997). For completeness it may be pointed out that, regarding ribose 3′-OH discrimination, T7 RNAP is similar to DNAPs like T7 DNAP which do not strongly discriminate against a hydrogen at the 3′-position and unlike DNAP I which does discriminate strongly against 3′-H groups (Axelrod & Kramer, 1985; Tabor & Richardson, 1995).

The mechanism in Figure 6 addresses the question of discrimination of the H-bonding character of the ribose 2′-group, but there must be other components to rNTP/dNTP discrimination since the Y639 mutants retain a residual preference for rNTPs and show a rank order preference for 2′-modified NTPs which differs from the wt enzyme. Our experiments do not allow us to determine whether other structural/chemical features of the 2′-substituent (volume, electronegativity) or conformational effects (ribose pucker) or some combination of these is responsible for the residual discrimination displayed by the Y639F mutants because these structural/conformational features are confounded in the NTP analogs used in these studies (Table 3). Another open question is why the less conservative Y639 mutants which are also less active should display increased rNTP/dNTP selectivity relative to Y639F (Table 1). Since all the Y639 mutants tested display loss of discrimination of the 2′-group H-bonding character, this does not appear to be explicable in terms of recovery of 2′-group H-bond character discrimination. Our observations are instead generally consistent with the following pattern: when the polymerization reaction is less efficient (relative to the high rates achieved by the wt or Y639F polymerases during the elongation reaction), the poor utilization of noncognate NTPs is exacerbated. This could be due to steps which are more sensitive to structural differences between dNTPs and rNTPs becoming rate limiting in the less active mutants or to increased steric conflicts between NTPs of inappropriate structure and active sites whose geometries have been disrupted by mutation. The latter explanation was invoked to explain the observation that some active site mutations in DNAP I give rise to dNTPαS elemental effects which are considerably larger than the maximal effects expected from the differential hydrolysis rates of phosphate and sulfur diesters (Polesky *et al*., 1992). However, it does not appear that disruption of active site/template/substrate geometry through mutation can offer a complete explanation for all our observations since we find that the "initiation reaction" (formation of the first phosphodiester bond and dinucleotide production) of the wt enzyme is more sensitive to the use of noncognate substrates

and even to the utilization of an ATP vs a GTP in the +2 position (Table 8). Such effects may find some explanation in the nonenzymatic, template-directed, metal-catalyzed polynucleotide synthesis reaction studied by Orgel and coworkers (Inoue & Orgel, 1983). One current view of polymerase mechanism is that the role of the active site in catalysis is limited to positioning the template base, primer/transcript terminus, NTP, and two metal ions with the correct geometry for caalysis (Steitz *et al*., 1994). This raises the possibility that the enzyme-catalyzed mechanism of bond formation is not intrinsically distinct from the nonenzymatic mechanism studied by Orgel and colleagues. If so, then the intrinsic reactivity of different nucleotides might becomes manifest with poorly active mutant enzymes or at inefficient points in the synthesis reaction (initial bond formation). Inoue and Orgel (1983) found that the most robust nonezymatic synthesis was poly(G) synthesis on poly(C) [or poly(dC)] templates. It may be relevant that the poly(G) synthesis reaction by T7 RNAP is more robust with respect to active site mutation than synthesis of heterogeneous sequence transcripts (Table I; Bonner *et al*., 1994; Mookhtiar *et al*., 1991) and that T7 RNAP preferentially initiates with three consecutive guanosines (changes in this initially transcribed sequence reduce initiation efficiency; Milligan *et al*., 1987, and Table 8). It could be that when optimal catalytic positioning of the NTP is compromised either by active site mutations or during initiation (when positioning of the 3′-NMP may be loose because an extended transcript has not been formed), the intrinsic ability of GTP to achieve proper geometry through hydrogen-bonding and stacking interactions may become important for efficient catalysis. Along similar lines, Lohrmann and Orgel (1977) found that rNTPs were more reactive than dNTPs in nonenzymatic reactions because development of negative charge on either the ribose 3′-OH or 2′-OH group during nucleophilic attack can be stabilized by hydrogen bonding between these groups. Such intrinsic differences in reactivity may only become manifest when the active site of the polymerase no longer contributes its full catalytic power. Indeed, preliminary experiments reveal that Y639V—but not Y639F or the wt enzyme—extends a 3′-dNMP terminated transcript with much poorer efficiency than a 3′-rNMP terminated transcript (Huang *et al*., unpublished observations). These considerations are not presented as explanations for all of our observations but are illustrative of the complexities involved. The unresolved nature of these issues need not impact the limited conclusion of our study regarding the mechanism of recognition of the H-bonding character of the ribose 2′-group and the role of desolvation at Y639 in limiting the net contribution to binding energy of the Y639 OH−2′-OH interaction.

It may be notable that recent observations bearing on base fidelity mechanisms in DNAPs and RT are consistent with the idea that polymerase active sites may be designed to minimize the contribution of polymerase−NMP moiety interactions to NTP binding, so that fidelity and NTP analog discrimination are often achieved indirectly, through template contacts (Beard *et al*., 1996; Carrol *et al*., 1991; Astatke *et al*., 1995; Tantillo *et al*., 1994). Structures of polymerase−NTP complexes have also revealed that polymerase−phosphate contacts make the primary polymerase contribution to securing the NTP in the active site (Beese *et al*., 1993; Pelletier et al., 1994), an observation which harkens back to the studies of Englund, Kornberg, and colleagues, who found that triphosphate binds more tightly to DNAP I than does a dNDP or dNMP (Englund *et al*., 1969) and led these workers to suggest that the dNTP binding site be dubbed the "triphosphate binding site". In terms of binding energy this designation may be apt, and it is possible that this reflects the different fates of the two products of catalysis: pyrophosphate and the 3′-NMP of the product nucleic acid. Release of the former from the active site is favored by a dilution effect, while the latter remains at high effective concentration in the vicinity of the active site throughout processive synthesis. It may make sense for a polymerase to design its active site to minimize affinity for a reaction product which is present at high effective concentration and hence to minimize affinity for that component of the substrate structure which corresponds to that reaction product. However, this line of reasoning makes sense only if the 3′-NMP of the product nucleic acid actually competes with binding of the NTP during processive synthesis, and this in turn has implications for the mechanism of polymerase translocation.

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